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Research Article

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Experimental and theoretical study of the influence of water on hydrolyzed product formation during the feruloylation of vegetable oil

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Abstract

BACKGROUND: Feruloylated vegetable oil is a valuable green bioproduct that has several cosmeceutical applications associated with its inherent anti-oxidant and ultraviolet-absorption properties. Hydrolyzed vegetable oil by-products can influence product quality and consistency.

RESULTS: The formation of by-products by residual water in the enzymatic synthesis of feruloylated vegetable oil was investigated using chemical theory and experimental studies by monitoring the reaction over a 22-day period. The hydrolysis of vegetable oil is thermodynamically favored over the hydrolysis of the ethyl ferulate starting material. These results suggest that hydrolyzed vegetable oil products will be experimentally observed in greater concentrations compared to hydrolyzed ethyl ferulate products.

CONCLUSION: Quantum chemical studies identified several reaction mechanisms that explain the formation of side products by water, suggesting that residual water influences product quality. Efforts to reduce residual water can improve product consistency and reduce purification costs.

Keywords: antioxidant; vegetable oil; ferulic acid; density functional theory; lipase

INTRODUCTION

Vegetable oils (as well as fruit oils) and plant extracts are major sources of green cosmeceuticals, which comprise an established area of products for those who wish to age naturally and healthily.[1,2] The pharmacological effects of bioactive oils can be enhanced through the rational modification of the oils to bioproducts with beneficial functional groups.[3,4] These intentional and unintentional transformations of vegetable oils provide value added bioproducts and govern product quality.[5] For example, biofuels and other value added bioproducts have been synthesized through the use of enzyme modified magnetic nanocomposites and microspheres, as well as catalytic functionalized mesoporous silicas.[6–8] Managing these reactions towards beneficial products can be aided by understanding the mechanisms and thermodynamics of unintentional products during synthesis. In addition, information gained by a detailed assessment of side-products can assist in the further refinement and increased yields in value-added product synthesis.[9] To this end, we carried out a series of quantum chemical calculations on transition states and products of the feruloylation of vegetable oil in the presence of the lipase enzyme, Novozym 435 (EC 3.1.1.3).

Phenyl propenoic acids and their esters (e.g. cinnamic, ferulic, coumaric, sinapic) are found ubiquitously throughout the plant kingdom as the monomeric constituents of lignin and as components of hemicelluloses present in the suberin and cutin waxy surfaces of leaves and other plants. Phenyl propenoic moieties provide natural antioxidative protection against lipid oxidation in plants[10,11] and natural protection against ultraviolet radiation (UV), as shown in plants along the perimeter of Antarctica.[12,13] Our research group has been interested in the transformation of vegetable oils to UV absorbing and anti-oxidant cosmeceutical ingredients.[14] The associated anti-oxidant and UV absorbing properties of ferulic acid makes this chemical moiety a desirable addition to vegetable oils.[15,16] Indeed, several feruloylated intermediates that synthetically lead to anti-oxidant derivatives of vegetable oil based bioproducts are found in nature, including 1-feruloyl-*sn*-glycerol, 1,2-diferulyol-*sn*-glycerol and 1,3-diferuloyl-*sn*-glycerol.[17–19] However, the cost of isolating

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these compounds as natural extracts is high and prohibitive to direct commercialization.

Enzymatic processes have been developed to incorporate the ferulate moiety into economical oils, and these economical products exhibit a variety of beneficial properties that are of interest to the personal care industry.[15,16,20] As a consequence, the enzymatic transesterification of phenyl propenoic esters with various vegetable oils has been the subject of much recent study.[21–26] Recently, we reported the commercial, pilot scale, continuous, packed-bed, enzymatic bioreactor production of feruloylated soybean oil.[16] However, although the production process is economically viable, the formation of side products can reduce quality and result in costly post production purifications. A detailed analysis of the mechanisms of formation of the observed side products is lacking.

In the present study, we address this knowledge gap by carrying out a detailed quantum chemical analysis of the side products of the transesterification of vegetable oil with ethyl ferulate aiming to gain insight into the mechanisms that help minimize side product formation. It was found that water can promote the formation of complex reaction mixtures. These results suggest that reduced water will minimize side product formation and increase yields of the desired feruloylated vegetable oil products.

MATERIALS AND METHODS

Materials

Non-genetically modified organism (GMO) soybean oil was obtained from Columbus Vegetable Oil (Des Plaines, IL, USA). The initial profile of the non-GMO soybean oil starting material was characterized by gas chromatography[16] and the results are shown in Table 1. The free fatty acid content was 0.0711 ± 0.0023% ($n = 3$). The Lovibond color was: yellow 2.0, red 0.0. Overall, the starting material principally consisted of mono- and di-unsaturated C18 fatty acids. Ethyl ferulate was obtained from Capot Chemicals Co., Ltd (Hangzhou, Zhejiang, China). Novozym 435 (*Candida antarctica* lipase B immobilized on acrylic beads) was obtained from Brenntag Great Lakes, LLC (Wauwatosa, WI, USA).

Table 1. Fatty acid profile of soybean oil

Fatty acid	Soybean oil (%)
C6:0	–
C8:0	–
C10:0	–
C12:0	–
C14:0	–
C16:0	10.87 ± 0.06
C18:0	3.83 ± 0.01
C18:1	21.58 ± 0.07
C18:2	53.53 ± 0.15
C18:3	7.96 ± 0.02
C20:0	0.32 ± 0.01
C22:0	0.38 ± 0.01
C24:0	–
Other	1.71 ± 0.02
Mol Wt	873.38

Results were presented as mean ± SD ($n = 5$ trials)
Mol Wt is the average molecular weight (g mol^{-1}) of the vegetable oil determined by the relative quantity of fatty acids.

Experimental for feruloylated soybean oil

Non-GMO soybean oil (500 g, 0.57 mol) and ethyl ferulate (100 g, 0.45 mol) were mixed in a 1-L Schlenk flask and heated at 60 °C under vacuum to dry overnight. The dried soybean oil-ethyl ferulate solution was analyzed by Karl Fisher titration (<25 ppm water) and the solution was transferred to a 2-L baffled Erlenmeyer shake flask.[16] Novozym 435 (75 g) was added, the shake flask was stoppered and the headspace flushed with dry nitrogen for 15 min. The flask was shaken at 60 °C and 115 rpm for 21 days in an Innova 4080 Incubator Shaker (Eppendorf Inc.; formerly New Brunswick Scientific, Hauppauge, NY, USA). The oil product was collected by vacuum filtration through a double layer of Whatman 54 filter paper (Fisher Scientific, Pittsburgh, PA, USA) using a Buchner funnel (135 x 60 mm) and 2-L collection flask. The recovered Novozym 435 was used for the subsequent reactions. Aliquots of the products from the shake reactions were analyzed by high-performance liquid chromatography using previously published methods.[16] The first soybean oil-ethyl ferulate transesterification reaction conditioned the enzyme and data from the three subsequent reactions were used for the basis of the modeling study.

Quantum chemistry

The results reported were obtained using the PM3 semi-empirical method, which was previously reported to be successful when calculating reaction reliable reaction coordinates for hydrolysis and esterification reactions.[27–29] Initial structures were built using HyperChem, version v8.0.10 (http://www.hyper.com) and optimized using the PM3 semi-empirical method and the Polak–Ribiere conjugate gradient. OpenBabel, version 2.3.0 (http://openbabel.org) was used for file transformation. The final results were calculated in Spartan '14, version 1.1.8 (https://www.wavefun.com) using the Baker's geometry optimization method. Results are reported using the B3LYP three parameter density functional theory method and the 6-31 + G* basis set. The SCF energy convergence criteria for the geometry optimization calculations was set at a maximum gradient tolerance of 3×10^{-4} hartrees bohr^{-1} and 1×10^{-6} hartree energy criteria, and a maximum distance tolerance of 1.2×10^{-3} Å. Thermodynamic properties were obtained using analytical gradients on frequencies from geometry optimized or transition state structures at 298.15 K and 1.00 atm.

RESULTS AND DISCUSSION

High quality, high yield chemistry is important for the commercialization of bioproducts. The reaction of ethyl ferulate, **1**, and vegetable oil, **2**, forms three value added bioproducts of interest (Fig. 1). The primary product is the mono-feruloylated vegetable oil derivative FDAG, **3**. Also, the di-feruloylated F_2MAG, **4**, and mono-feruloylated FMAG, **5**, are formed that exhibit beneficial bioactivity profiles for cosmeceuticals and sun screens. The product FMAG, **5**, is of chemical interest because it has a hydroxylzed glyceride at the β-position. This observed hydrolysis is somewhat surprising because of efforts made to dry the reagents. The hydrolysis raises questions about the role of residual water in the reaction. Most lipases require a minimum amount of water to retain activity.[30,31] In laboratory, bench-scale, packed-bed bioreactor studies, Novozym 435 was shown to exhibit a 'bell-shaped' curve relationship between water content and activity towards ethyl ferulate in soybean oil with the maximum lipase activity obtained at approximately 250 ppm water.[32] In pilot-scale,

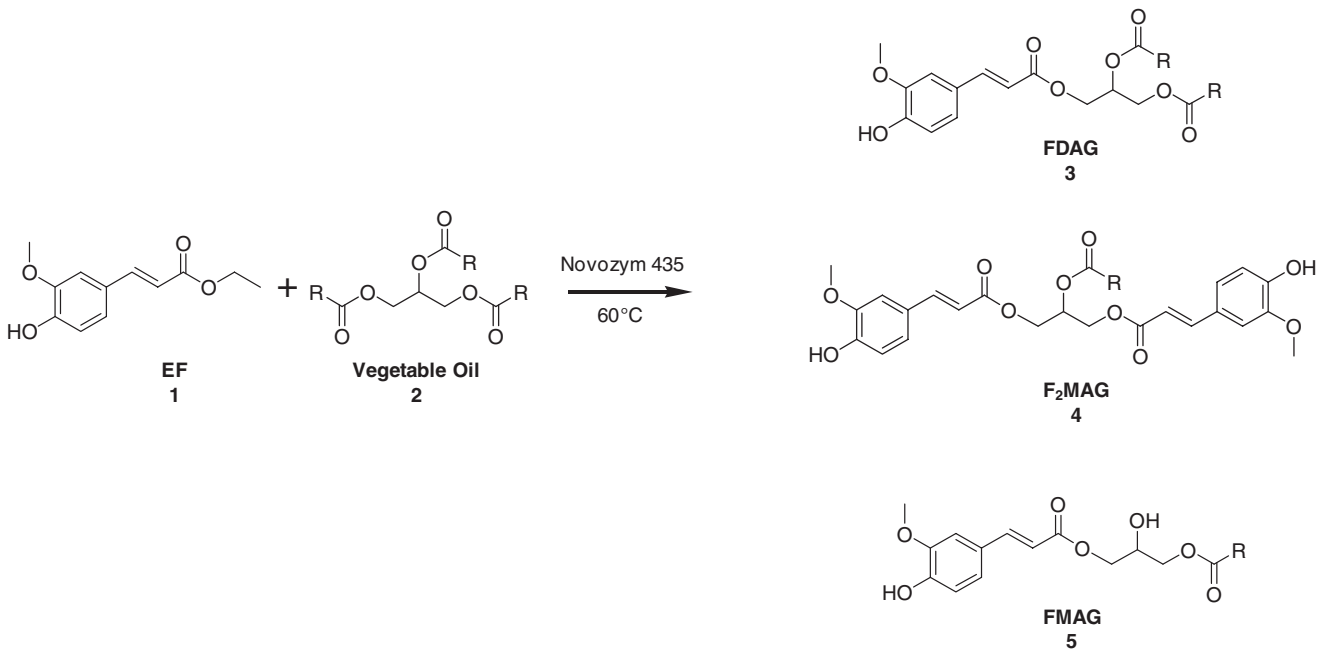

Figure 1. Reactants and products of the feruloylation of vegetable oil. R, fatty acid.

continuous, packed-bed enzyme bioreactor production, it was demonstrated that the residual water from the Novozym 435 as obtained from the manufacturer (10–20 g kg^{-1}) was sufficient to support enzyme activity for up to 5 months of continuous feruloylated soybean oil production (60–65% conversion) when the ethyl ferulate-soybean oil substrate was dried to < 25 ppm water.[16] The extremely low water content was shown to limit the formation of unwanted hydrolysis side products, as discussed below, and was the condition examined in the present study. However, some moisture content is necessary for the enzyme to function. The optimal temperature and water activity for pilot scale production were used in the present study and have been reported previously.[32,33] To obtain insight into the impact of residual water on the formation of desired and undesired hydrolyzed species, a quantum chemical study was carried out aiming to investigate the reaction coordinates of hydrolysis.

The enzymatic transesterification of vegetable oil and ethyl ferulate resulted in the desired, lipophilic species, **3–5** (Fig. 1) that possessed the UV absorbing/antioxidant feruloyl moiety, as well as a long chain fatty acid that provided water resistance in cosmeceutical applications.[20] The undesired, hydrolyzed products, which lack a fatty acid group and are more hydrophilic, are shown in Fig. 2. FG, **6**, is the feruloylated derivative of vegetable oil that is hydrolyzed in the α- and β-positions. F_2G, **7**, is di-feruloylated and hydrolyzed in the β-position, and ferulic acid (FA), **8**, is the hydrolyzed derivative of ethyl ferulate (EF), **1**. Undesired side-products **6–8**, can precipitate out at low concentrations and adversely affect product quality and consistency. Certain phenolic acid side products, such as FA, **8**, have shown to have adverse sensory effects.[34] The undesired products **6–8** are susceptible to formation from the reagents and products in the presence of water.

Vegetable oil is a triglyceride extract from a plant with varying fatty acid side chain composition. To make our theoretical study manageable, a general analog of vegetable oil was used. The simplest triglyceride, 1,2,3 propantriol triacetate, contains all the important functional groups necessary to describe the reaction chemistry of vegetable oil and was selected to represent vegetable oil. In addition, the reduced chain length allowed the quantum based calculations to be more manageable.

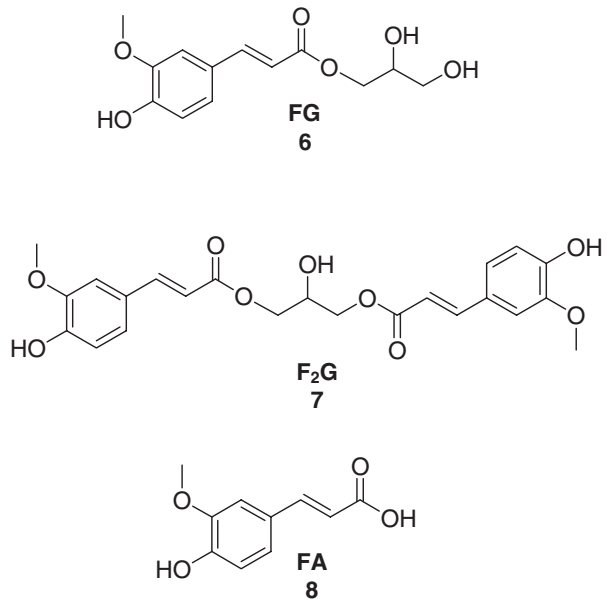

Figure 2. Undesired side products of the feruloylation of vegetable oil.

The quantum level results are provided in Tables 2 and 3, and the structures are shown in Fig. 3. It is interesting to note that there is considerable variation in the dipole of the reagents and products (1.75–5.49 eV). Overall, hydrolysis of ethyl ferulate, **1**, to ferulic acid, **8**, reduces entropy. The transesterification generally increases entropy for feruloylated vegetable products compared to the intrinsic entropy of the reagents. It is also interesting to note that the HOMO-LUMO gap obtained from the electronic structure calculations *in vacuo* is approximately 4 eV for all feruloylated species. This suggests that transesterification and hydrolysis of ethyl ferulate and the feruloylated products has a minimal effect

Table 2. Energies and properties reagents and products of feruloylation of ethyl ferulate (1) with 1,2,3 propanetriol triacetate (2)

Compound	EF	Vegetable	FDAG	F2MAG	FMAG
	1	2	3	4	5
E (kJ mol^{-1})	−2012771.7	−2107695.7	−3312607.1	−4517413.2	−2911783.7
E_{aq} (kJ mol^{-1})	−2012796.5	−2107740.6	−3312651.1	−4517481.1	−2911832.7
E_{solv} (kJ mol^{-1})	−24.8	−44.9	−44.5	−68.0	−49.0
Dipole (ev)	1.75	5.48	4.41	3.17	5.36
ZPVE (kJ mol^{-1})	637.1	610.3	933.5	1260.7	835.0
H (kJ mol^{-1})	−2011939.1	−2107047.0	−3311616.7	−4516077.2	−2910897.9
S (J mol^{-1})	478.3	483.9	613.5	729.4	569.4
Cv (J mol^{-1})	180.0	175.2	275.4	377.4	244.7
G (kJ mol^{-1})	−2012239.2	−2107191.3	−3311799.6	−4516294.7	−2911067.7
€$_{LUMO}$ (eV)	−1.87	−0.63	−2.02	−2.06	−2.14
€$_{HOMO}$ (eV)	−5.94	−7.80	−6.06	−5.98	−6.14

Table 3. Energies and properties side-products of feruloylation of ethyl ferulate (1) with 1,2,3 propanetriol triacetate (2)

Compound	FG	F_2G	FA
	6	7	8
E (kJ mol^{-1})	−2510949.1	−4116631.7	−1806341.5
E_{aq} (kJ mol^{-1})	−2510998.3	−4116694.4	−1806378.3
E_{solv} (kJ mol^{-1})	−49.2	−62.7	−36.8
Dipole (ev)	5.49	2.30	2.96
ZPVE (kJ mol^{-1})	736.5	1160.2	488.3
H (kJ mol^{-1})	−2510168.0	−4115402.2	−1805821.0
S (J mol^{-1})	524.9	692.2	436.7
Cv (J mol^{-1})	214.3	345.8	154.5
G (kJ mol^{-1})	−2510324.5	−4115608.6	−1805951.2
€$_{LUMO}$ (eV)	−2.12	−2.08	−2.04
€$_{HOMO}$ (eV)	−6.13	−5.92	−6.06

on the excitation properties and electrophilic reactivity of these species.

Several hydrate schemes were considered to investigate the reaction coordinate for the hydrolysis of ethyl ferulate and vegetable oil. In Fig. 4, ethyl ferulate,**1**, reacts with a molecule of water to form the hydrated ester. This hydrated ester results in fusaric acid and ethanol. Figure 5 provides the schemes for the hydrolysis of vegetable oil at the α- and β-positions. For each scheme, the ester hydrate is formed, followed by the fatty acid chain leaving. The result is a partially hydrolyzed triglyceride and fatty acid chain.

The reaction coordinate for the hydrolysis of ethyl ferulate to fusaric acid is provided in Fig. 6. The hydrolysis goes through two transition states. The first involves formation of the hydrate ester and the second transition state entails the removal of ethanol. The reaction coordinate for the hydrolysis of vegetable oil is provided in Figs 7 and 8. Figure 7 provides the two transition states and hydrate ester for the hydrolysis of the vegetable oil derivative at the α-position and Fig. 8 provides the species for the hydrolysis at the internal β-position. It should be noted that the triglyceride possess twice the number α-fatty acids to β-fatty acid residues; thus, hydrolysis at the α-position is twice as likely to occur compared to at the β-position.

Considering the hydrolysis reaction coordinates ethyl ferulate and vegetable oil, it becomes apparent that the hydrolysis of the vegetable oil is favored thermodynamically compared to the ethyl ferulate. Also, the hydrolysis of the triglyceride product is exergonic by over 16–25 kJ mol^{-1} for the both the α-fatty acids to β-fatty acid position. By contrast, the hydrolysis of ethyl ferulate is exergonic by 2.5 kJ mol^{-1}.

To clarify the influence of residual water on product formation of the reaction of ethyl ferulate with vegetable oil in the presence of Novozym 435, the reaction was monitored for 22 days with the products quantitified using liquid chromatography on days 0, 1, 3, 5, 7, 19, 14, 18 and 22. The results are provided in Table 4. Ethyl ferulate, **1**, is reacted with neat vegetable oil, **2**, in the presence of Novozym 435 at 60 °C. The desired product FDAG, **3**, is formed over time, as well as the di-feruloylated F_2MAG, **4**, product and hydrolyzed product FMAG, **5**. However, several hydrolyzed side products, **6–8**, are formed during the synthesis that are difficult to explain without a role of water. Residual water is present in the driest commercially available vegetable oils (∼150 ppm) and the ethyl ferulate contained 50 g kg^{-1} water as obtained from the manufacturer, making it necessary to dry the ethyl ferulate-soybean oil substrate (< 25 ppm water to limit unwanted hydrolysis side products. The enzyme Novezym 435 contains residual water molecules as well (10–20 g kg^{-1}) as obtained from the manufacturer, and as discussed above.

The main products of the transesterification are desired products FDAG, **3**, and the diferuloylated derivative F_2MAG, **4**. Both of these products lack hydrolyzed functional groups. After 1 day, the products are less than 19% of the reaction mixture, and consist of almost 65% of the reaction mixture. Throughout the entire reaction, the presence of the ethyl ferulate, **1**, hydrolyzed product ferulic acid, **3**, is less than 0.5%. The vegetable oil hydrolyzed products constitute between 3.2% and 3.8% of the products. Albeit, the standard deviation of the formation of these products suggests their formation does not have a significant influence on product consistency (±1.4%). The levels of the side products are associated with the reaction conditions that have been optimized for the desired products. These reaction conditions include the transformations catalyzed by the Novozym 435 enzyme. It should be noted that a temperature of 60 °C is the manufacturer's recommended temperature for maximum enzyme activity and longevity. Increasing the temperature will increase the kinetic activity at the cost of life of the enzyme. It is interesting to note that the dihydroxlyzed product FG, **6**, is not observed during the time course of the reaction at 60 °C. The formation of FG requires more water for hydrolysis at two sites of the triglyceride. The lack of FG formation suggests the factors that promote the hydrolysis of triglyceride are

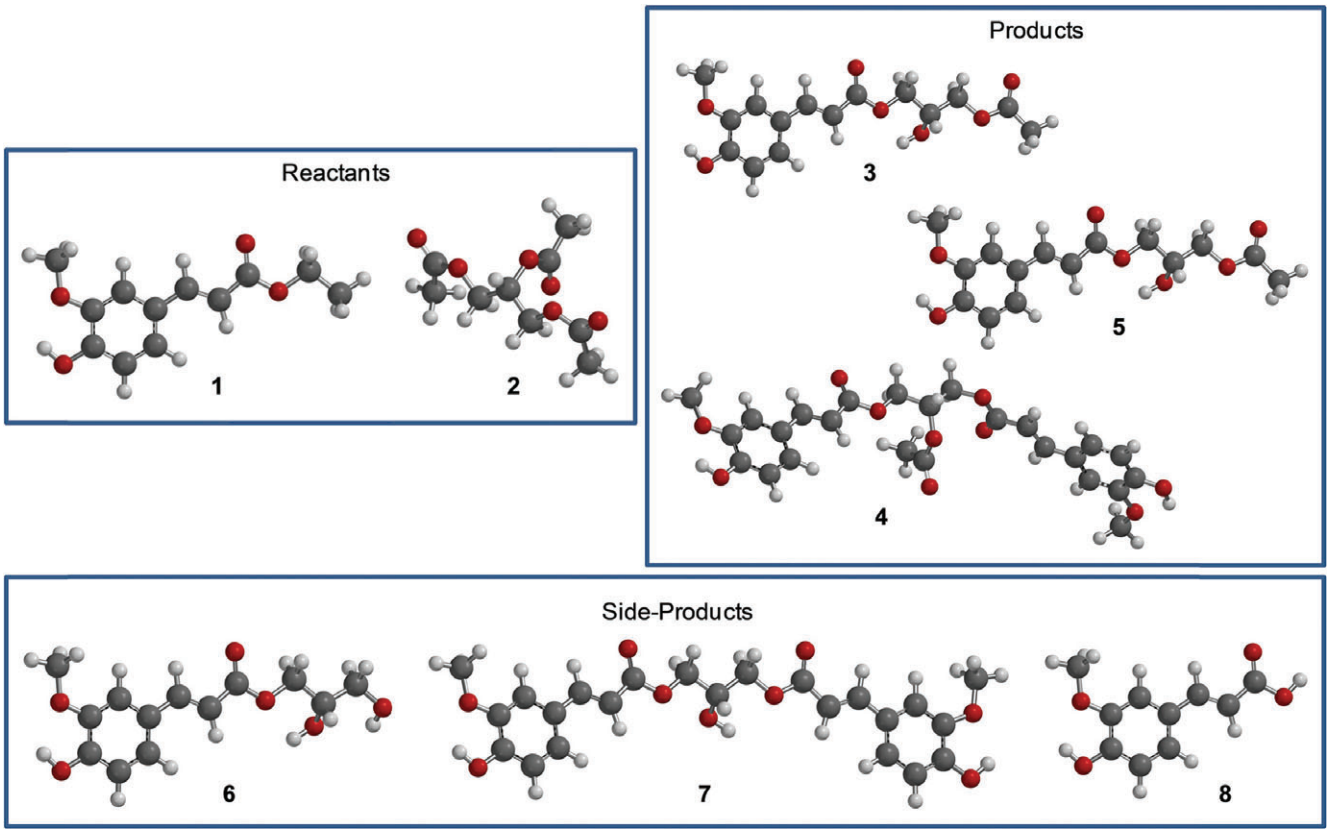

Figure 3. Geometry optimized structures of reactants, products and side-products.

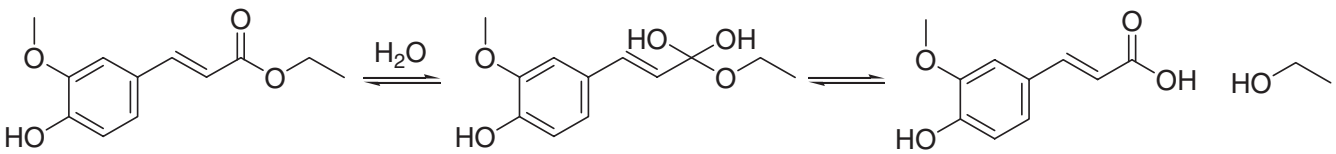

Figure 4. Hydrolysis of ethyl ferulate in the presence of water.

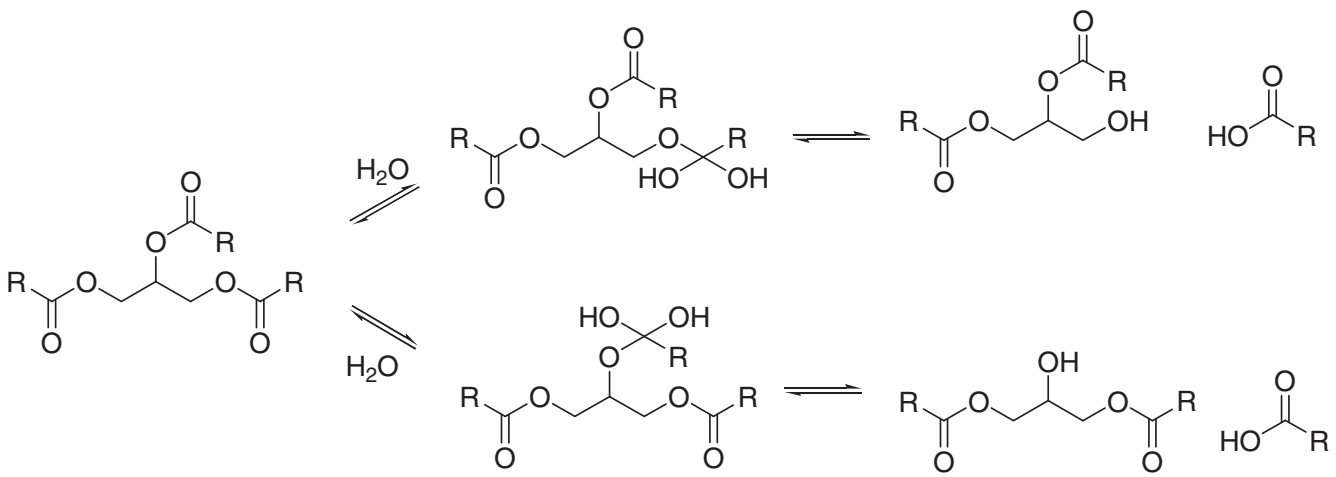

Figure 5. Hydrolysis of vegetable oil in the presence of water.

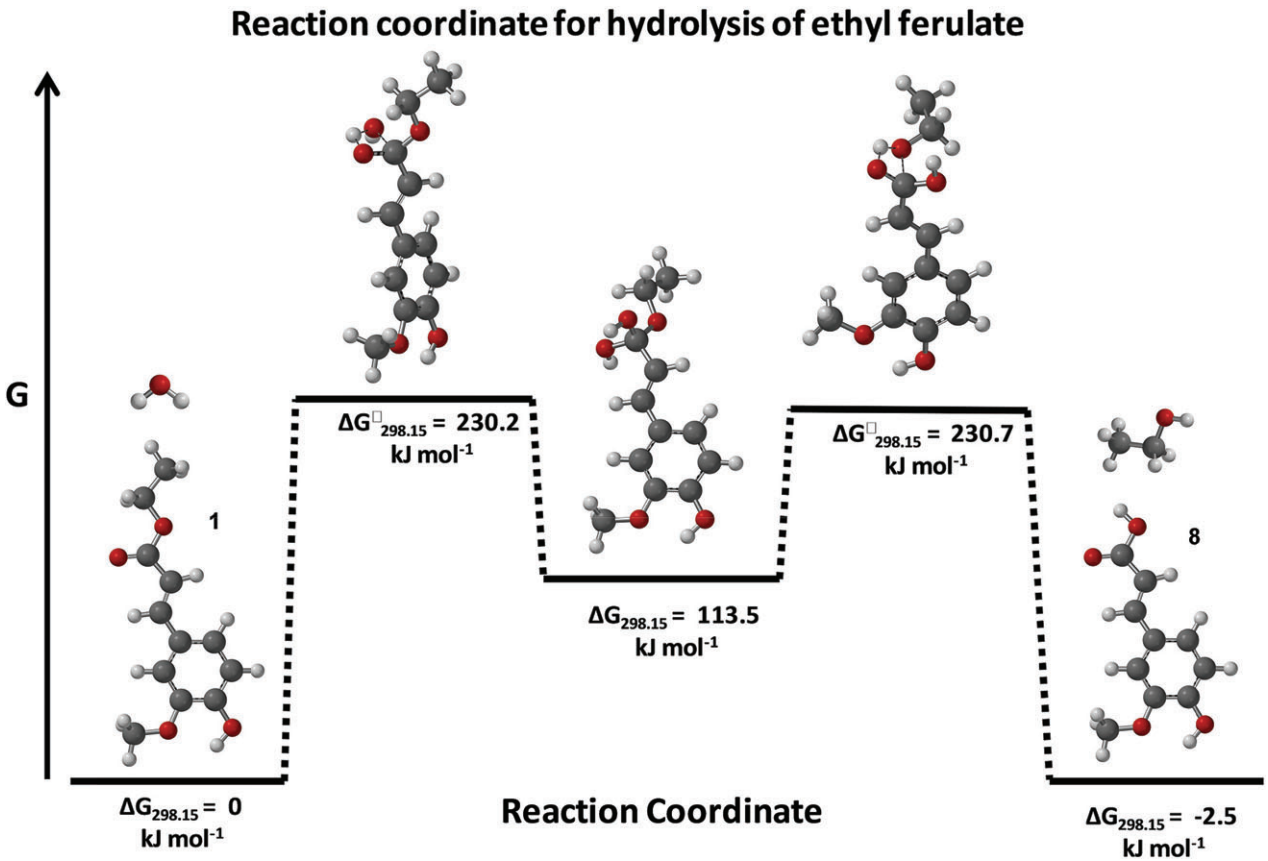

Figure 6. Reaction coordinate for hydrolysis of ethyl ferulate.

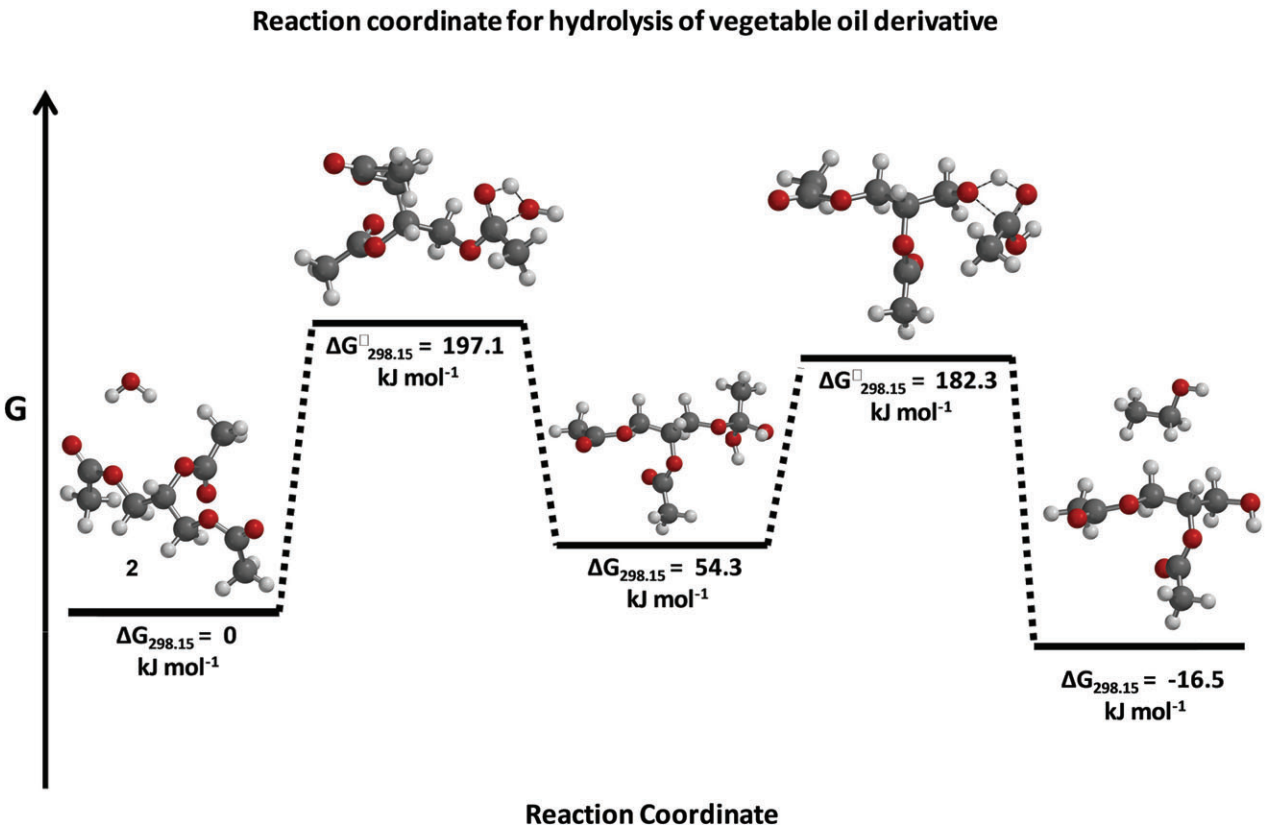

Figure 7. Reaction coordinate for hydrolysis of triglyceride acid adduct.

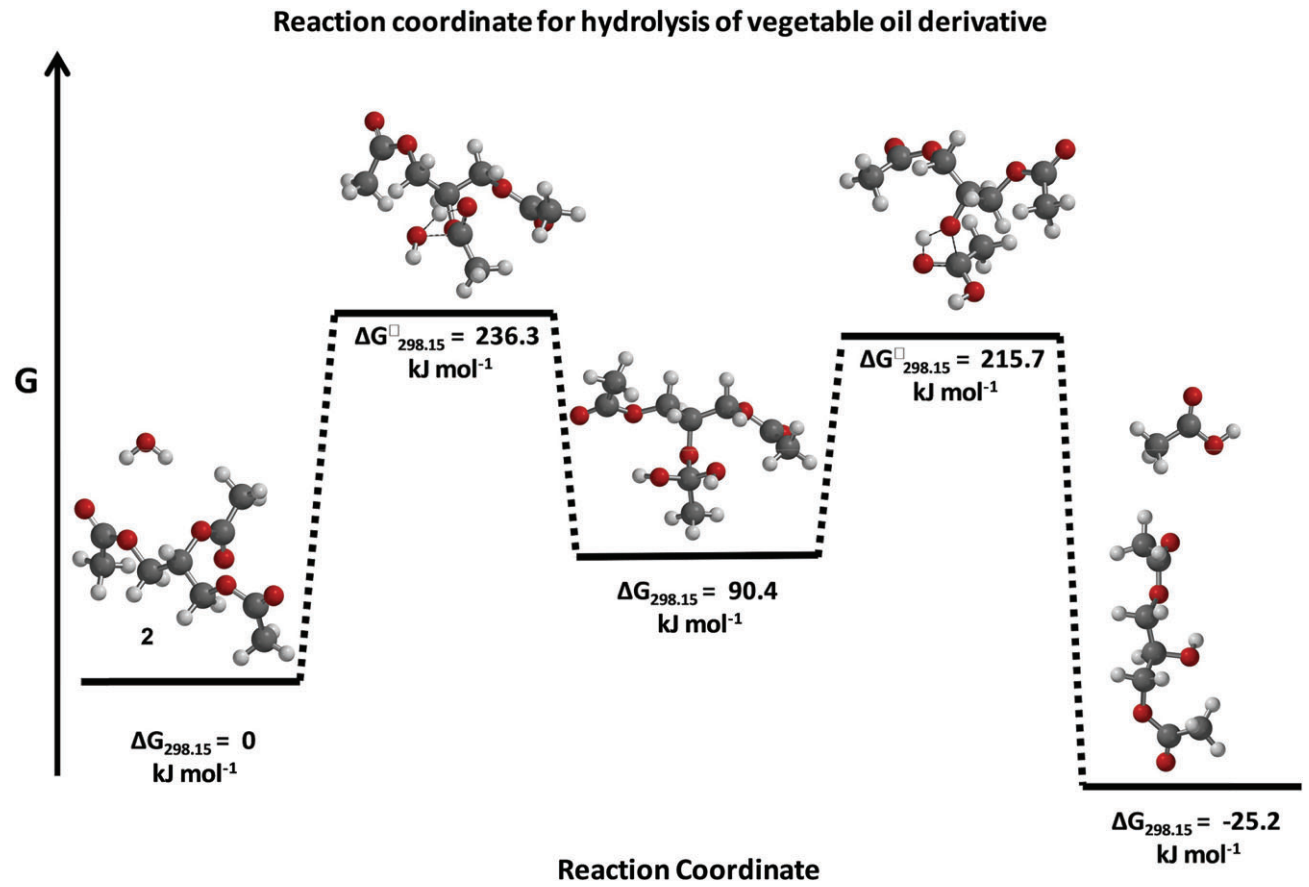

Figure 8. Reaction coordinate for hydrolysis of a vegetable oil analog at the internal position.

Table 4. Observed relative abundance (%) of products formed during the enzymatic transesterification of vegetable oil with ethyl ferulate as determined by high-performance liquid chromatography

Compound	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14	Day 18	Day 22
1 EF	100 ± 0	78.1 ± 2.1	63.0 ± 5.1	53.0 ± 6.7	47.3 ± 6.8	40.4 ± 6.5	35.5 ± 4.8	29.8 ± 9.8	27.4 ± 8.3
3 FDAG	0	11.4 ± 2.3	20.4 ± 2.8	27.6 ± 4.4	31.4 ± 4.6	37.1 ± 5.7	41.2 ± 4.4	42.4 ± 2.8	45.1 ± 1.8
4 F2MAG	0	7.3 ± 1.0	12.8 ± 0.8	15.6 ± 0.8	17.5 ± 0.6	18.7 ± 0.6	19.6 ± 1.5	20.0 ± 1.3	19.7 ± 1.6
5 FMAG	0	2.4 ± 0.8	2.7 ± 0.9	2.6 ± 0.9	2.8 ± 1.0	3.0 ± 0.9	3.0 ± 1.1	3.0 ± 1.1	3.2 ± 1.4
6 FG	0	0	0	0	0	0	0	0	0
7 F_2G	0	0.8 ± 0.4	0.7 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.3
8 FA	0	0	0.3 ± 0	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	0.3 ± 0.3	0.4 ± 0.4

Data are the mean ± SD of three trials.

minor components of the reaction mixture when using typically dry reagents.

CONCLUSIONS

Understanding the formation of hydrolysis products in the synthesis of feruloylated vegetable oil from ethyl ferulate can improve the resulting product quality. Chemical theory studies on the hydrolysis products of reagents used in the synthesis of feruloylated vegetable oil revealed that triglyceride hydrolysis is thermodynamically favored. In addition, triglyceride hydrolysis at the α-position possesses lower barriers of hydrolysis compared to the hydrolysis of ethyl ferulate. Experimentally, common drying techniques are suitable for reducing side product formation for the enzymatic transesterification synthesis of feruloylated vegetable oil without loss of lipase activity.

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